# Exposure of Fibrinogen Receptors on Fresh and Stored Platelets by ADP and Epinephrine as Single Agents and as a Pair

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**Platelet concentrates stored at 22°C have a marked decrease in their aggregation response to adenosine diphosphate (ADP) or epinephrine but a normal response to these agents when used as a pair. Since platelet stimulation involves exposure of receptors for fibrinogen, we studied fibrinogen binding to platelets from fresh and stored concentrates. Following stimulation with 10 $\mu M$ ADP or 20 $\mu M$ epinephrine, platelet suspensions from fresh concentrates bound $^{125}$I-fibrinogen in a reaction that reached completion within 30 min. Significantly less binding occurred in suspensions from platelet concentrates that had been stored for 5 days at 22°C. When stimulated by ADP and epinephrine as a pair (2 $\mu M$ each), binding of fibrinogen to platelets was complete within 10–15 min and was not significantly decreased in suspensions from stored concentrates. We also investigated the effect of storage on the glycoprotein IIb–IIIa complex, thought to be a specific receptor for fibrinogen on the platelet surface. Binding of a monoclonal antibody specific for this complex (B59.2) to platelet suspensions was unaffected by 5 days of storage. Furthermore, B59.2 inhibited aggregation, secretion, and fibrinogen binding of fresh and stored platelets stimulated with the pair of agents just as it did with single agents. We conclude that storage for 5 days at 22°C impairs the exposure of fibrinogen receptors on platelets in response to ADP or epinephrine when used as single agents, without affecting the glycoprotein IIb–IIIa complex quantitatively. The function of the receptor is normal in response to the pair of agents.**

PLATELETS STORED at 22°C for 3–5 days, although functionally useful after transfusion in vivo,[1-4] progressively lose their ability to respond in vitro to single aggregating agents. The most striking changes are seen when adenosine diphosphate (ADP) or epinephrine are used.[5-8] However, stored platelets retain full aggregation potential in response to low concentrations of these two agents as a pair.[8] It has been shown[9,10] that ADP and epinephrine act synergistically to induce exposure of fibrinogen receptors on fresh human platelets. Fibrinogen binding to platelets is important for platelet aggregation, since washed platelets do not aggregate in response to ADP or epinephrine[11-16] and platelets in platelet-rich plasma (PRP) from afibrinogenemic patients aggregate poorly in response to a variety of aggregating agents.[17] Therefore, we have investigated the exposure of fibrinogen receptors in response to ADP and epinephrine either employed singly or as a pair in platelet suspensions from freshly prepared platelet concentrates (PC) and from PC stored for 5 days at 22°C. We report that platelets from PC stored for 5 days bind fibrinogen like those from fresh PC in response to low concentrations of ADP and epinephrine in combination, although the response to these agents used alone is reduced. This suggests that storage affects the function, but not the number, of fibrinogen receptors.

To confirm this conclusion, we have also made observations on changes occurring during storage in the glycoprotein IIb–IIIa (GpIIb–IIIa) complex, which is thought to be at least one of the receptors for fibrinogen on the platelet surface.[18-23] To do this, we have utilized a monoclonal antibody, B59.2, which is specific for this complex. In a recent publication,[24] we reported that this antibody precipitated the GpIIb–IIIa complex from solubilized platelet membranes, bound to a single class of binding sites on whole platelets, and inhibited aggregation, secretion, and fibrinogen binding of platelets in response to ADP, collagen, and arachidonic acid. We find no change in the GpIIb–IIIa content of stored platelets. Furthermore, B59.2 inhibits the platelet response to a pair of agents just as it does to single agents.

## MATERIALS AND METHODS

Blood from eight normal volunteers who had not taken any drugs for at least 10 days before venipuncture was collected into citrate-phosphate-dextrose-adenine (CPD-A). PRP and PC were prepared as previously described.[7] The PC platelet count was adjusted to 1000–1200 × $10^9$/liter with the platelet-poor supernatant from PC preparation to prevent a fall in pH and loss of viability during storage.[4] Platelet counts were obtained using a Coulter Counter as previously described.[25] PC were stored at 22°C in commercially available transfer packs constructed of plastic, PL-146 (Fenwal Corp., Morton Grove, IL) on a horizontal agitator[7] for 5 days. The pH was measured at 22°C as previously described.[25] After 5 days of storage, the lowest pH observed was 6.95. Platelet-poor plasma

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(PPP) was prepared by centrifugation of PRP in an Eppendorf 3200 centrifuge (Brinkman Instruments, Inc., Westbury, NY) at 12,000*g* for 5 min.

Aggregating agents employed were ADP (adenosine-5 diphosphate, sodium salt, Sigma Co., St. Louis, MO) and epinephrine (epinephrine hydrochloride, isotonic solution, Elkins-Sinn, Inc., Cherry Hill, NJ). Dilutions of the agents were in Tris buffer, 0.154 *M*, pH 7.4. Silicon oils (methyl silicon 1.0 DC 200 and Hi phenyl silicon 125 DC 550) were purchased from W. F. Nye, Inc., Specialty Lubricants, New Bedford, MA. Mixtures of DC 200 and DC 550 were prepared as described by Niewiarowski et al.[26] Disodium ethylenediaminotetracetate (EDTA) was obtained from Fisher Scientific Company, King of Prussia, PA. Bovine serum albumin (Pentex, fraction V) was purchased from Miles Laboratories Inc., Elkhart, IN.

Suspensions of washed platelets were prepared as previously described[24] from aliquots of PC on the day of their preparation and after 3 and 5 days of storage at 22°C. The methods for measurement of platelet aggregation and secretion and the binding of $^{125}$I-fibrinogen and the monoclonal antibody, B59.2, to platelets in these suspensions have been reported in detail previously.[24]

Statistical analysis was performed using Student's t test for paired comparisons. Informed consent was obtained from the donors after approval of the local Human Investigation Committee, in accordance with an assurance filed with and approved by the H.S.S.

## RESULTS

### *Effect of Storage on $^{125}$I-Fibrinogen Binding to Platelets*

As shown in Fig. 1, following stimulation with 10 $\mu M$ ADP, a time-dependent binding of fibrinogen to fresh platelets occurred in a reaction that was complete within 15–30 min. Fibrinogen binding to platelets stimulated by 20 $\mu M$ epinephrine approached an apparent plateau after 15 min. However, further binding occurred between 15 and 60 min. Similar results, both quantitatively and qualitatively, were obtained when 100 $\mu M$ ADP or 200 $\mu M$ epinephrine were the stimuli employed. When the combination of 2 $\mu M$ ADP and 2 $\mu M$ epinephrine was the stimulus, binding of fibrinogen to platelets reached a maximum within 10–15 min. Similar results, both quantitatively and qualitatively, were obtained when 100 $\mu M$ ADP and 200 $\mu M$ epinephrine in combination were the stimulus used. EDTA (10 m$M$) inhibited fibrinogen binding to platelets in response to 10 $\mu M$ ADP, 20 $\mu M$ epinephrine, or 2 $\mu M$ ADP and epinephrine as a pair by 84.7% ± 1.3%, 85.6% ± 2.1%, and 84.9% ± 1.9%, respectively. After 15 min of exposure to all three stimuli, 84%–87% of labeled fibrinogen could be displaced by a 100-fold excess of cold protein. However, after 30 min, 37%–45% of the binding was irreversible.

Storage at 22°C for 5 days did not alter the patterns of fibrinogen binding that had been observed in suspensions from fresh PC (Fig. 1), although the amounts bound were changed. This affinity of fibrinogen for stimulated platelets was studied quantitatively by incubating platelet suspensions with increasing amounts (from 3 n$M$ to 1,200 n$M$) of labeled fibrinogen for 30 min when ADP or epinephrine were employed alone and for 15 min when these agents were used as a pair. $K_d$ and number of receptors at high and low affinity sites were determined by Scatchard analysis as previously described.[24] The results of these experiments (Table 1) indicated that storage for 5 days at 22°C significantly decreased the number of low-affinity binding sites for fibrinogen exposed in response to 10 $\mu M$ ADP or 20 $\mu M$ epinephrine, whereas it did not significantly affect binding in response to low concentrations of ADP and epinephrine used together. Similar results were obtained when 100 $\mu M$ ADP and 200 $\mu M$ epinephrine were employed either singly or in combination (data not shown).

### *Binding of Monoclonal Antibody B59.2*

Binding of $^{125}$I-antibody B59.2 to unstimulated platelets was complete in 1 min and quantitatively similar in suspensions from fresh and 5-day stored PC (Table 2). The number of molecules bound was not significantly reduced by 5 days of storage ($p > 0.05$).

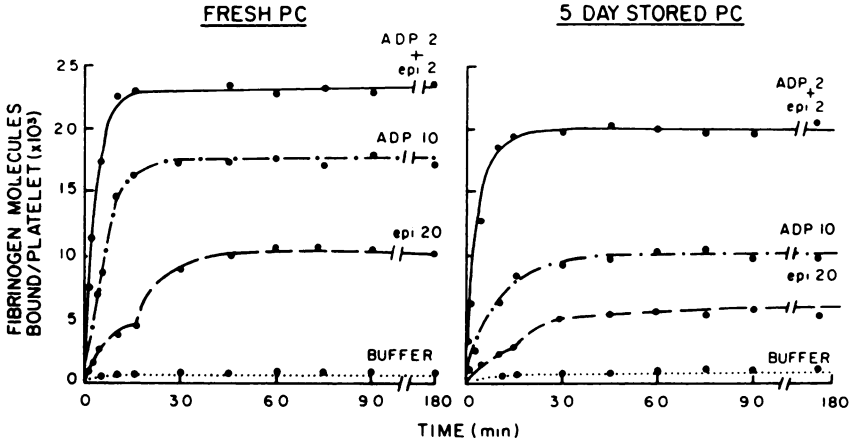


**Fig. 1. Time-course of $^{125}$I-fibrinogen binding to platelets. Aliquots of 0.5 ml of unstirred platelet suspensions (5 × 10$^8$/ml) were incubated at room temperature with 10 $\mu M$ ADP, 20 $\mu M$ epinephrine, or the combination of 2 $\mu M$ ADP and 2 $\mu M$ epinephrine. After 3 min, 25 $\mu$l of a 0.5 $\mu M$ concentration of $^{125}$I-fibrinogen was added. At time intervals between 1 and 180 min, 0.4-ml aliquots of the mixture were layered onto 50 $\mu$l silicon oil and centrifuged for 2 min at 12,000*g*. The free and platelet-bound $^{125}$I-fibrinogen were counted separately. The data reported are the means of 8 determinations.**

Table 1. $^{125}$I-Fibrinogen Binding to Platelets From Suspensions of Fresh and Stored PC (Mean ± SEM of 8 Determinations)

| | High-Affinity Sites | | Low-Affinity Sites | |
|---|---|---|---|---|
| | $K_d$ ($\mu M$) | $n$ | $K_d$ ($\mu M$) | $n$ |
| ADP (10$\mu M$) | | | | |
| Fresh PC | 0.026 | 1,202 ± 482 | 1.2 | 27,920 ± 3,970† |
| 3-Day stored PC | 0.026 | 1,050 ± 328 | 1.1 | 20,230 ± 4,012 |
| 5-Day stored PC | 0.025 | 992 ± 477 | 1.1 | 15,612 ± 3,215* |
| Epinephrine (20$\mu M$) | | | | |
| Fresh PC | 0.025 | 1,030 ± 320 | 1.08 | 15,208 ± 2,730‡ |
| 3-Day stored PC | 0.025 | 1,009 ± 296 | 1.08 | 9,978 ± 3,490 |
| 5-Day stored PC | 0.025 | 756 ± 579 | 1.08 | 7,416 ± 2,978* |
| ADP (2$\mu$M) + epinephrine (2$\mu M$) | | | | |
| Fresh PC | 0.026 | 1,631 ± 221 | 1.2 | 44,971 ± 2,828 |
| 3-Day stored PC | 0.026 | 1,341 ± 498 | 1.2 | 43,631 ± 4,270 |
| 5-Day stored PC | 0.026 | 1,202 ± 501 | 1.2 | 41,678 ± 5,697 |

*$p < 0.01$, significant difference between stored and fresh PC.

†$p < 0.05$, significant difference between platelets stimulated by ADP or epinephrine alone and platelets stimulated by ADP and epinephrine together in suspensions from fresh PC.

‡$p < 0.01$, significant difference between platelets stimulated by ADP or epinephrine alone and platelets stimulated by ADP and epinephrine together in suspensions from fresh PC.

All other comparisons are not statistically significant ($p > 0.05$)

### *Aggregation and Secretion in Washed Platelet Suspensions From Fresh and Stored PC*

In the absence of added fibrinogen, washed platelet suspensions from freshly prepared PC did not aggregate in response to 100 $\mu M$ ADP or 200 $\mu M$ epinephrine, whereas they showed partial aggregation and secretion in response to 2 $\mu M$ ADP and 2 $\mu M$ epinephrine as a pair (Table 3). Aggregation and secretion did not occur in suspensions from PC stored for 3 or 5 days in response to this pair of agents in the absence of fibrinogen.

In the presence of fibrinogen, suspensions from fresh PC showed full aggregation and secretion of adenosine triphosphate (ATP) in response to 10 $\mu M$ ADP. No detectable aggregation or secretion could be found in response to concentrations of epinephrine as high as 200 $\mu M$. In suspensions from fresh PC, a 2 $\mu M$ concentration of ADP, which only caused partial aggregation when used alone, caused full aggregation and secretion of ATP when used in combination with 2 $\mu M$ epinephrine. The rate and extent of aggregation and secretion in response to ADP and epinephrine in pairs was not significantly affected by 5 days of storage at 22°C, whereas aggregation in response to 10 $\mu M$ ADP alone was reduced by more than 46% and secretion was totally abolished (Table 3).

Table 2. Binding of $^{125}$I-B59.2 to Unstimulated Platelet Suspensions (Mean ± SEM of 8 Determinations)

| | B59.2 Binding | |
|---|---|---|
| | $K_d$ (n$M$) | Molecules Bound/Platelet |
| Fresh PC | 19.2 | 21,324 ± 3,312 |
| 5-Day stored PC | 19.0 | 19,891 ± 4,791 |

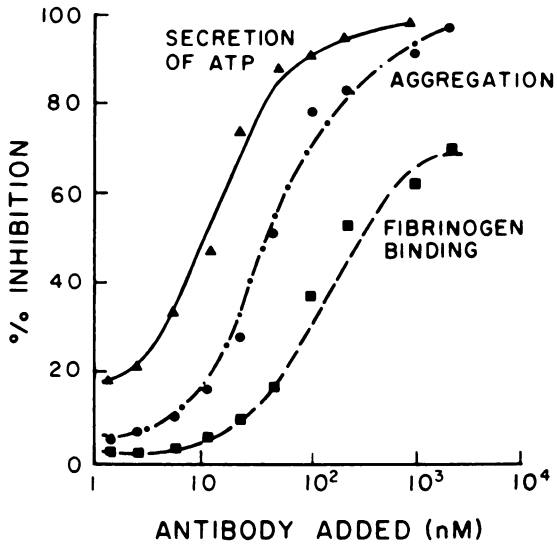


**Fig. 2. Dose-dependent inhibition by antibody B59.2 of platelet aggregation, secretion, and fibrinogen binding of platelet suspensions from 5-day stored PC stimulated with 2 $\mu M$ ADP and 2 $\mu M$ epinephrine in combination. Increasing concentrations of B59.2 were incubated with 0.5-ml platelet suspension (5 × 10$^8$/ml) that had been stirred at 37°C for 1 min at 1,000 rpm. After 1 min, 25 $\mu$l unlabeled fibrinogen (0.5 $\mu M$ final concentration) was added, and 1 min later, 50 $\mu$l of the mixture of firefly luciferase and luciferin were added, followed by the addition of microliter amounts of each aggregating agent. Aggregation and secretion were quantitated as described previously.[24] In parallel, aliquots of 0.5 ml of unstirred platelet suspensions (5 × 10$^8$/ml) were incubated at room temperature with identical concentrations of aggregating agents. After 3 min, increasing concentrations of B59.2 were added, and 1 min later, $^{125}$I-fibrinogen (0.5 $\mu M$ final concentration) was added. After 15 min, free and platelet-bound fibrinogen were separated on silicon oil and counted. The data reported are means of 8 determinations. It should be emphasized that the inhibition of fibrinogen binding is underestimated, because at 15 min, 14% of the bound labeled fibrinogen could not be displaced by a 100-fold excess of the cold protein.**

**Table 3. Aggregation and ATP Secretion of Washed Platelets From Suspensions of Fresh and 5-Day Stored PC**
**(Mean ± SEM of 8 Determinations)**

| | Fresh PC | | 5-Day Stored PC | |
|---|---|---|---|---|
| | Aggregation (LTU)* | ATP Secretion ($\mu M$) | Aggregation (LTU) | ATP Secretion ($\mu M$) |
| ADP (10$\mu M$) + fibrinogen (0.5$\mu M$) | 54.3 ± 7.2 | 1.7 ± 0.5 | 29.9 ± 2.1† | ND |
| Epinephrine (20$\mu M$) + fibrinogen (0.5$\mu M$) | ND | ND | ND | ND |
| ADP (2$\mu M$) + epinephrine (2$\mu M$) + buffer | 19.7 ± 3.1 | 0.9 ± 0.1 | ND | ND |
| ADP (2$\mu M$) + epinephrine (2$\mu M$) + fibrinogen (0.5$\mu M$) | 56.7 ± 4.2 | 1.8 ± 0.7 | 50.6 ± 5.2 | 1.7 ± 0.6 |

*LTU, light transmission units.
†$p < 0.01$ significant difference between aggregation of fresh and 5-day stored platelet suspensions in response to 10 $\mu M$ ADP.
ND, not detectable.

### *Inhibition of Platelet Aggregation, Secretion, and Fibrinogen Binding by B59.2*

We have previously shown[24] that B59.2 inhibited aggregation, secretion, and fibrinogen binding of fresh platelets stimulated by ADP. Similarly, for both fresh and stored platelets, increasing concentrations (from 1.2 n$M$ to 2.1 $\mu M$, final) of B59.2 inhibited, in a dose-dependent fashion, aggregation, secretion, and fibrinogen binding of platelets stimulated by 2 $\mu M$ ADP and 2 $\mu M$ epinephrine as a pair. The data for stored platelets are shown in Fig. 2. The rate and extent of inhibition of fibrinogen binding to fresh platelets stimulated with ADP and epinephrine as a pair were similar to those seen in platelet suspensions exposed to 10 $\mu M$ ADP or 20 $\mu M$ epinephrine as single agents (Table 4). No significant difference in the pattern and the extent of inhibition of fibrinogen binding was found in suspensions from fresh and 5-day stored PC (Table 4).

## DISCUSSION

It has previously been reported that freshly collected platelets expose fibrinogen receptors following stimulation with a variety of aggregating agents.[9,10,18-22,24,26] We have extended these observations to demonstrate similar properties for 5-day stored PC. The rate and the patterns of fibrinogen binding to suspensions from PC stimulated by ADP and epinephrine in pairs were found to be comparable to those reported by others[9,10] on suspensions from freshly collected platelets. Compared to fresh PC, we have found a significant reduction in the number of molecules of fibrinogen bound to suspensions from 5-day stored PC after exposure to 10 $\mu M$ ADP or 20 $\mu M$ epinephrine. However, full exposure of fibrinogen receptors was observed when suspensions from 5-day stored PC were exposed to low concentrations of ADP and epinephrine as a pair. The results make it unlikely that abnormalities of receptors for ADP or epinephrine are involved in the defect with these agonists as single agents after storage, since the

**Table 4. Effect of 2.1$\mu M$ B59.2 on $^{125}$I-Fibrinogen Binding to Suspensions From Fresh and 5-Day Stored PC**
**(Mean ± SEM of 8 Determinations)**

| | | High-Affinity Sites | | Low-Affinity Sites | |
|---|---|---|---|---|---|
| | | $K_d$ ($\mu M$) | $n$ | $K_d$ ($\mu M$) | $n$ |
| ADP (10$\mu M$) | | | | | |
| Fresh PC | Buffer | 0.026 | 1,202 ± 482 | 1.2 | 27,920 ± 3,970 |
| | B59.2 | 0.025 | 1,016 ± 378 | 1.1 | 9,651 ± 6,013* |
| 5-Day stored PC | Buffer | 0.025 | 992 ± 477 | 1.1 | 15,612 ± 3,215 |
| | B59.2 | 0.025 | 1,003 ± 401 | 1.1 | 5,232 ± 2,001* |
| Epinephrine (20$\mu M$) | | | | | |
| Fresh PC | Buffer | 0.025 | 1,030 ± 320 | 1.08 | 15,208 ± 2,730 |
| | B59.2 | 0.025 | 1,112 ± 279 | 1.08 | 6,003 ± 2,920* |
| 5-Day stored PC | Buffer | 0.025 | 756 ± 579 | 1.08 | 7,416 ± 2,978 |
| | B59.2 | 0.025 | 910 ± 501 | 1.08 | 2,098 ± 621* |
| ADP (2$\mu M$) + epinephrine (2$\mu M$) | | | | | |
| Fresh PC | Buffer | 0.026 | 1,631 ± 221 | 1.2 | 44,971 ± 2,828 |
| | B59.2 | 0.025 | 1,782 ± 603 | 1.2 | 15,413 ± 465* |
| 5-Day stored PC | Buffer | 0.026 | 1,202 ± 501 | 1.2 | 41,678 ± 5,697 |
| | B59.2 | 0.026 | 1,082 ± 633 | 1.2 | 13,938 ± 2,797* |

*$p < 0.01$ significant difference between antibody-treated and buffer-treated platelet suspensions.

response to the pair is intact. These data correlate with our previous observation[8] that stored platelets, which are completely unresponsive to 200 $\mu M$ epinephrine and markedly insensitive to 100 $\mu M$ ADP, retain full in vitro aggregation in response to low concentrations of these two agents as a pair. The previous observation was made with fresh and stored platelets suspended in plasma. In this study, we obtained the same result with washed platelets (Table 3).

We further analyzed our finding by examining the platelet GpIIb–IIIa complex, thought to be at least one of the receptors for fibrinogen on the platelet surface.[18-24,26] Using a monoclonal antibody to this complex, B59.2, we found no quantitative reduction in this complex after storage. This is in agreement with what others found,[27] employing densitometric scanning of periodic acid-Schiff (PAS) stained gels of suspensions from fresh and stored PC. These results suggest that the fibrinogen receptor is present and functional on stored platelets, but that its exposure by some single agents, such as ADP or epinephrine, is impaired.

These data have implications for the mechanisms of aggregation induced by pairs of agents. The kinetics of fibrinogen binding in response to the pair of ADP and epinephrine are similar to those seen with single agents. Furthermore, antibody B59.2 inhibited aggregation, secretion, and fibrinogen binding of platelets stimulated with ADP and epinephrine as a pair just as it does when the agents are used singly. These data suggest that ADP and epinephrine as a pair expose the same fibrinogen receptors on platelets as either of these agents employed alone. In the absence of added fibrinogen, suspensions from fresh PC aggregated in response to ADP and epinephrine used as a pair (Table 3). However, this aggregation response was substantially less than that found when fibrinogen was added along with the aggregating agents. These data indicate that, as for ADP or epinephrine used singly, fibrinogen plays a major role in the aggregation of platelets in response to ADP and epinephrine as a pair.

We have found that 2 $\mu M$ ADP in combination with 2 $\mu M$ epinephrine is able to cause aggregation and secretion in washed platelet suspensions from fresh PC in the absence of added fibrinogen. It is possible that secretion of $\alpha$-granules supplied the necessary fibrinogen to support aggregation. On the other hand, the pair of agents was unable to produce aggregation or secretion in washed platelet suspensions from PC stored for 5 days without added fibrinogen. It has previously been shown[28] that $\alpha$-granular components are released by platelets during storage. There may be insufficient $\alpha$-granular fibrinogen after storage to be released to support aggregation.

We conclude that storage for 5 days at 22°C impairs the exposure of fibrinogen receptors on platelets in response to ADP or epinephrine without affecting the glycoprotein IIb–IIIa complex quantitatively. The function of the receptor is normal in response to the pair of agents. It appears that the pair of agents acts by exposing the same fibrinogen receptors as single agents. These results with fibrinogen binding correlate with our previous data showing that aggregation of platelets with ADP and epinephrine as a pair after storage is normal, while there is markedly decreased response to each as a single agent.

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